Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation

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Glucocorticoids acting through the glucocorticoid receptor (GR) inhibit TNF-induced lethal inflammation. Here, we demonstrate that GR dimerization plays a role in reducing TNF sensitivity. In mutant mice unable to dimerize GR, we found that TNF failed to induce MAPK phosphatase 1 (MKP1). We assessed TNF sensitivity in Mkp1−/− mice and found increased inflammatory gene induction in livers, increased circulating cytokines, cell death in intestinal epithelium, severe intestinal inflammation, hypothermia, and death. Mkp1−/− mice had increased levels of phosphorylated JNK, which promotes apoptosis, in liver tissue. We further examined JNK-deficient mice for their response to TNF. Although Jnk1−/− mice showed no change in sensitivity to TNF, Jnk2−/− mice were significantly protected against TNF, identifying JNK2 as an essential player in inflammation induced by TNF. Furthermore, we found that loss of Jnk2 partially rescued the increased sensitivity of Mkp1−/− and mutant GR mice to TNF. Our data show that GR dimerization inhibits JNK2 through MKP1 and protects from TNF-induced apoptosis and lethal inflammation.

Introduction

TNF plays an important role in inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease (IBD), and psoriasis (1, 2). Biological drugs that inhibit TNF are effective, despite some limited side effects (3). However, the mechanism by which TNF activates and sustains the inflammatory response is not yet clear. TNF induces transcription factors, such as NF-κB and AP-1, that stimulate the expression of hundreds of genes, activate cell death, and cause tissue damage (4). Clinical and animal studies have identified hypotension, liver toxicity, and bowel necrosis as the major determinants of TNF lethality (5).

Both exogenous and endogenous glucocorticoids (GCs) protect against TNF-induced lethal inflammation (6, 7). The binding of GCs to the GR receptor (GR) protects against TNF lethality, as shown by the use of GR blockers and GR-deficient mice (8, 9). However, the protective mechanism is not fully elucidated. GC-activated GR can form dimers, which bind to GC response elements (GREs) (1) and lead to transactivation (TA) of metabolic and anti-inflammatory genes. GR can also transrepress genes by binding as a monomer to transcription factors such as NF-κB and AP-1 (10, 11). It has been proposed that the antiinflammatory effects of GR are mainly mediated by transrepression (TR) and that the induction of unwanted side effects of GC therapy (such as type 2 diabetes) is mediated by TA. Therefore, researchers are developing ligands that favor GR monomers (12, 13). However, several transactivated genes that have been identified encode strong anti-inflammatory molecules, such as MKP1 (14, 15). MKP1 (encoded by Mkp1; also known as dual-specificity phosphatase-1, Dusp1) is a member of the family of dual-specificity phosphatases, which preferentially dephosphorylate MAPKs, such as p38 and JNK (16). Since these MAPKs are essential in the initiation of inflammation, MKP1 has been described as critical for the resolution of inflammation. Indeed, Mkp1−/− mice are very sensitive in models of endotoxemia, sepsis, and other inflammatory diseases (17–19).

To identify the protective mechanism of endogenous GCs and GR, we here used mice expressing a mutant version of the GR protein, namely GRdim/dim. This mutant protein carries a single point mutation (A458T) that prevents it from forming dimers (20, 21). Hence, GRdim/dim mice are largely devoid of TA induced by GR dimers and are more sensitive to inflammatory diseases, such as contact hypersensitivity and sepsis (22, 23). These findings indicate that at least 1 GRE gene is induced by GR and has antiinflammatory functions in the GRdim/dim model, and perhaps in other models and diseases as well.

In this work, we used GRdim/dim mice to study the contribution of GRE genes to the protection against the lethal effects of TNF. We concluded that endogenous GCs protected against TNF lethality in a GR dimer–dependent manner. This protection specifically involved MKP1-mediated dephosphorylation of the proapoptotic JNK2 MAPK (24, 25).

Results

GRdim/dim mice are extremely sensitive to TNF and fail to induce MKP1. Whether the TA potential of GR is necessary for its antiinflammatory activity is somewhat controversial. In this study, we investigated whether the induction of GRE genes is necessary for the anti-inflammatory functions counteracting TNF lethality. We injected WT control (GRwt/wt) and GRdim/dim mice i.p. with 25 μg TNF (a nonlethal dose for FVB/N mice) and monitored survival and body temperature. Mortality rate was significantly higher and hypothermia more pronounced in GRdim/dim than in GRwt/wt mice (Figure 1, A and B). Because IL-6 level is a good indicator of TNF sensitivity (26, 27), we measured IL-6 protein levels in circulation.
0.5 hours after TNF stimulation. The significantly higher IL-6 levels in GRdim/dim mice (Figure 1C) confirmed their hypersensitivity to TNF. Furthermore, H&E staining of ileum samples showed that TNF treatment resulted in more severe intestinal damage in GRdim/dim than in GRwt/wt mice (Figure 1D). These data showed that dimerization of GR was essential for protection against TNF-induced shock, presumably by the induction of antiinflammatory GRE genes by endogenous GCs.

One of the most potent antiinflammatory genes induced by GR is Mkp1. To evaluate Mkp1 expression in our setting, GRwt/wt and GRdim/dim mice were injected i.p. with 500 μg of a synthetic GC, dexamethasone (DEX), or with PBS. Liver samples were isolated 1 hour after DEX treatment, and mRNA expression of Mkp1 and Sgk1, a well-known GR dimer–dependent TA gene, was measured by quantitative real-time PCR (qPCR). DEX treatment of GRwt/wt mice resulted in a strong induction of Mkp1 and Sgk1 expression in liver; this induction was significantly weaker in GRdim/dim mice (Figure 1D). Because Mkp1 regulation by GCs is not restricted to the transcriptional level, MKP1 protein levels were also measured. MKP1 protein was induced after DEX injection only in GRwt/wt mice (Figure 1E). These data showed that dimerization of GR was indispensable for the induction of Mkp1 and subsequent induction of this gene indeed require GR dimerization.

Furthermore, as it is known that inflammatory stimuli can also induce Mkp1, most likely via the production of endogenous GCs, we investigated the induction of Mkp1 after TNF treatment. We injected GRwt/wt and GRdim/dim mice i.p. with 25 μg TNF and harvested liver samples after 0.5 hours. In GRwt/wt mice, TNF treatment resulted in the induction of Mkp1, whereas in GRdim/dim mice, this induction was significantly reduced (Figure 1F). These data indicate that GR dimerization is necessary for the induction of Mkp1 by DEX or by endogenous GCs induced by TNF. Taken together, our data demonstrated that dimerization of GR was indispensable for protection against TNF lethality. Furthermore, our findings provided evidence that Mkp1 is a GR dimer–dependent gene that might be involved in this protection against TNF.
in Mkp1^{−/−} mice (Figure 2, A and B). These findings indicate that MKP1 plays a crucial role in controlling TNF lethality. To investigate the underlying mechanism, Mkp1^{−/−} and Mkp1^{+/−} mice were injected i.p. with 5 μg TNF, and blood, liver, and ileum samples were obtained 0, 1, and 6 hours after TNF treatment. We measured IL-6 protein in circulation and liver mRNA levels 0, 1, and 6 hours after 5 μg TNF (n = 6 per group). (C and D) Mice were injected i.p. with 5 μg TNF, and 0, 1, and 6 hours later, they were euthanized, and livers (D) and IECs (E) were isolated for qPCR analysis of Ccl5, Timp1 and Nos2 levels (n = 5 per group). (F) Standard H&E and active caspase 3 staining of ileum samples (n = 5 per group). Representative images are shown. The ileum was sampled 0 and 1 hour after injection of 5 μg TNF. The micrograph at the 0-hour time point is representative of both Mkp1^{+/−} and Mkp1^{−/−} mice. Scale bars: 100 μm. Original magnification, ×40. (C–F) *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 hours or as indicated by brackets. (G and H) Survival of Mkp1^{−/−} (G) and Mkp1^{+/−} (H) mice pretreated with 10 mg/kg DEX (squares; n = 8 per group) or solvent (circles; n = 8 [Mkp1^{+/−}]; 7 [Mkp1^{−/−}]) and injected with 15 μg (G) or 7.5 μg (H) TNF. *P < 0.01, DEX vs. solvent. (A–H) Black bars and symbols, Mkp1^{+/−}; white bars and symbols, Mkp1^{−/−}.

As it was recently shown that MKP1 inhibits TNF-induced endothelial barrier dysfunction and apoptosis in HUVECs (28), and since TNF can cause intestinal damage and enterocyte apoptosis (5, 29), we investigated acute cell death in ileum samples of Mkp1^{−/−} and Mkp1^{+/−} mice using H&E staining. As early as 1 hour after TNF challenge, the toxic effect of TNF was much more pronounced in Mkp1^{−/−} than in Mkp1^{+/−} mice (Figure 2F). The intestinal damage was mainly characterized by loosening of the lamina propria, erosion of the villi, and loss of goblet cells. The degree of intestinal damage was scored according to a previously published method (30). Intestines of Mkp1^{−/−} mice were significantly more damaged than those of control Mkp1^{+/−} mice (Figure 2F). Furthermore, ileum samples were stained for active caspase 3 as a marker of apoptosis. In agreement with the H&E staining, we found significantly more cells expressing active caspase 3 in ileum samples of Mkp1^{−/−} mice (Figure 2C). Several other cytokines and chemokines in circulation were also higher in Mkp1^{−/−} than in Mkp1^{+/−} mice, especially 6 hours after TNF treatment (Supplemental Figure 2). Additionally, we tested the expression levels of different TNF-induced proinflammatory genes in liver samples and in intestinal epithelial cells (IECs). Again, Mkp1^{−/−} livers had significantly higher mRNA levels of Ccl5 (encoding CCL5 or RANTES), Timp1 (encoding the MMP inhibitor TIMP1) and Nos2 (encoding iNOS) 6 hours after TNF treatment (Figure 2, D and E). Strikingly, Mkp1^{−/−} mice had significantly higher mRNA levels of these proinflammatory genes in IECs as early as 1 hour after TNF. These results indicated that MKP1 has an antiinflammatory effect that protects against TNF-induced lethal inflammation.

Figure 2
Mkp1^{+/−} mice are hypersensitive to TNF-induced lethality. (A and B) Survival (A) and body temperature (B) of Mkp1^{+/−} (n = 12) and Mkp1^{−/−} (n = 22) mice after injection of 5 μg TNF. *P < 0.05, **P < 0.001, Mkp1^{−/−} vs. Mkp1^{+/−}. (C) Serum IL-6 levels and liver I6 mRNA levels 0, 1, and 6 hours after 5 μg TNF (n = 6 per group). (D and E) Mice were injected i.p. with 5 μg TNF, and 0, 1, and 6 hours later, they were euthanized, and livers (D) and IECs (E) were isolated for qPCR analysis of Ccl5, Timp1 and Nos2 levels (n = 5 per group). (F) Standard H&E and active caspase 3 staining of ileum samples (n = 5 per group). Representative images are shown. The ileum was sampled 0 and 1 hour after injection of 5 μg TNF. The micrograph at the 0-hour time point is representative of both Mkp1^{+/−} and Mkp1^{−/−} mice. Scale bars: 100 μm. Original magnification, ×40. (C–F) *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 hours or as indicated by brackets. (G and H) Survival of Mkp1^{−/−} (G) and Mkp1^{+/−} (H) mice pretreated with 10 mg/kg DEX (squares; n = 8 per group) or solvent (circles; n = 8 [Mkp1^{+/−}]; 7 [Mkp1^{−/−}]) and injected with 15 μg (G) or 7.5 μg (H) TNF. *P < 0.01, DEX vs. solvent. (A–H) Black bars and symbols, Mkp1^{+/−}; white bars and symbols, Mkp1^{−/−}.

Figure 3
JNK phosphorylation in liver is higher in Mkp1^{+/−} and GRdim/dim mice. (A) Western blot analysis of phospho-JNK1/2 protein levels in livers of Mkp1^{−/−} and Mkp1^{+/−} mice. Mice were injected i.p. with 5 μg TNF, and livers were harvested at the indicated times after challenge. The phospho-JNK1/2 bands (46 kDa and 54 kDa) were normalized to the intensities of the total JNK1/2 and actin bands (42 kDa). Black bars, Mkp1^{+/−}; white bars, Mkp1^{−/−}. (B) Western blot analysis of phospho-JNK1/2 protein levels in livers of GRwt/wt and GRdim/dim mice. Mice were treated with 25 μg TNF; 0 and 0.5 hours later, they were euthanized, and livers were obtained for Western blot analysis. Normalized values are also shown. Black bars, GRwt/wt; white bars, GRdim/dim. (A and B) *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 hours or as indicated by brackets. See complete unedited blots in the supplemental material.

The Journal of Clinical Investigation
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Because phospho-JNK1/2 levels were significantly higher in livers of Mkp1+/− and GRdim/dim mice than in their WT controls, we studied the response of JNK-deficient mice to TNF. WT control, Jnk1−/−, and Jnk2−/− mice were injected i.p. with 10 μg TNF (an LD50 dose for C57BL/6 mice), and survival and body temperature were monitored. Although Jnk1−/− mice showed no change in sensitivity to TNF, Jnk2−/− mice were significantly more protected against TNF than control mice, as shown by the significantly lower mortality rate and less severe hypothermia (Figure 4, A and B). These findings indicated that JNK2, but not JNK1, is an essential mediator of TNF-induced lethal shock.

Furthermore, control and Jnk2−/− mice were injected i.p. with 10 μg TNF, and blood, liver, and ileum samples were harvested 0, 1, and 6 hours later. IL-6 protein and Il6 mRNA levels, cytokine and chemokine levels in circulation, and proinflammatory gene expression levels in liver clearly demonstrated that Jnk2−/− mice were protected against TNF-induced inflammation, especially at the later 6-hour time point (Figure 4, C and D, and Supplemental Figure 5). Together, these data indicated that JNK2 has a proinflammatory role.

Moreover, measuring proinflammatory gene expression levels in IECs and stainings of ileum samples with H&E and for active caspase 3 showed that inflammatory state, tissue damage, and acute cell death 1 hour after TNF injection was much more pronounced in the intestine of Jnk2−/− than in Jnk1−/− mice (Figure 4, E and F). As these characteristics were absent in Jnk2−/− mice, we hypothesized that JNK2 might be the mediator of TNF-induced intestinal permeability. We therefore injected Jnk2−/− and Jnk2+/− mice with 10 μg TNF, followed 3 hours later by oral administration of 25 mg/ml FITC-dextran. Blood samples were collected 8 hours after TNF challenge, and plasma was tested for FITC signal. Jnk2−/− mice showed a stronger signal than Jnk2+/− mice (Figure 4G), which indicates that TNF induced more intestinal permeability in the Jnk2−/− mouse. Taken together, these observations indicated that the effects of TNF on the epithelium (i.e., induction of inflammation and apoptosis) started very early, by 1 hour after TNF. Increased inflammation in liver and circulation seemed to be secondary to intestinal damage, as shown by the defect in intestinal permeability. Additionally, our findings provided evidence that JNK2 is a critical mediator of these TNF effects.

Mkp1+/− Jnk2−/− and GRdim/dim Jnk2−/− mice are less sensitive to TNF. Since Mkp1−/− mice were very sensitive to the in vivo effects of TNF, and since Mkp1 also dephosphorylated JNK1/2 in the TNF model, we wondered whether the sensitivity of Mkp1−/− mice to TNF is due to overactive JNK kinases. To test this hypothesis, we generated Mkp1−/− Jnk2−/− mice and studied their sensitivity to TNF. Control Mkp1−/− Jnk2+/−, Mkp1+/−, and Mkp1−/− Jnk2+/− mice were injected i.p. with 5 μg TNF (which is lethal for Mkp1−/− mice), and mortality and body temperature were monitored. The sensitivity of Mkp1−/− Jnk2+/− mice to TNF was intermediate between that of Mkp1−/− Jnk2+/− and Mkp1+/− mice (Figure 5, A and B), which suggests that Jnk2 rescues TNF sensitivity, at least in part, in Mkp1−/− mice. The intermediate sensitivity of Mkp1−/− Jnk2−/− mice was confirmed by measuring IL-6 protein in circulation and Il6 mRNA in liver (Figure 5C). Furthermore, H&E staining of ileum samples 1 hour after TNF injection showed that intestinal damage was comparable in Mkp1−/− Jnk2−/− and Mkp1+/− Jnk2−/− mice, but much more pronounced in Mkp1−/− mice (Figure 5D). These observations indicate that Mkp1−/− Jnk2−/− mice are much less sensitive to TNF than are Mkp1+/− mice, which indicates that the sensitivity of Mkp1−/− mice

Figure 4

Jnk2−/− mice are resistant to TNF-induced shock. (A) Survival of control (black squares; n = 19), Jnk2−/− (white squares; n = 12), and Jnk1+/− (gray circles; n = 6) mice after i.p. injection of 10 μg TNF. **P < 0.01 vs. control. (B) Body temperature of control (n = 12), Jnk2−/− (n = 12), and Jnk1−/− (n = 6) mice after injection of 10 μg TNF. **P < 0.05. (C) Serum IL-6 levels and liver Il6 mRNA levels 0, 1, and 6 hours after challenge with 10 μg TNF (n = 5 per group). (D) and (E) Mice were injected i.p. with 10 μg TNF; 0, 1, and 6 hours later, they were euthanized, and livers (D) and IECs (E) were obtained for qPCR analysis of Ccl5, Tip3p1, and NOS2 levels (n = 5 per group). (F) Standard H&E and active caspase 3 staining of ileum samples (n = 5 per group). Representative images are shown. The ileum was sampled 0 and 1 hours after TNF injection. The micrograph at 0 hours is representative of both control and Jnk2−/− mice. Scale bars: 100 μm. Original magnification, ×40. (C–F) *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 hours or as indicated by brackets. (G) Relative permeability 8 hours after injection of 10 μg TNF or PBS. **P < 0.01. (B–G) Black bars and symbols, control; white bars and symbols, Jnk2−/−; gray bars and symbols, Jnk1+/−.

(Figure 2F). These data indicate that within 1 hour after TNF treatment, Mkp1+/− mouse intestine is severely damaged because of a high rate of apoptosis combined with severe inflammation. Additionally, to investigate whether GC inhibition of the response to TNF occurs through MKP1, we examined whether pretreating Mkp1+/− mice with endogenous GCs can protect against TNF-induced lethal shock. We injected the mice with 10 mg/kg DEX and 0.5 hours later with LD50 of TNF (15 μg for Mkp1+/−; 7.5 μg for Mkp1−/−). In keeping with previous reports by our group (7, 9), Mkp1+/− mice were protected against TNF by pretreatment with DEX compared with mice pretreated with diluted methanol solvent (90% vs. 10% survival; Figure 2G). However, pretreatment of Mkp1+/− mice with DEX or solvent failed to protect them against TNF lethality (10% survival; Figure 2H). These data demonstrated that MKP1 was indispensable in the protective action of GCs against TNF.

Prolonged JNK activation in Mkp1−/− and GRdim/dim mice. MKP1 is known to dephosphorylate and hence inactivate MAPKs, particularly JNK and p38, but also ERK (16). As MAPKs are activated by TNF and mediate part of the TNF proinflammatory induction profile (31), we examined the status of activated MAPKs in liver samples of Mkp1+/− and Mkp1−/− mice. Both mouse groups were injected i.p. with 5 μg TNF and sacrificed at time point 0 or after 10 minutes or 0.5, 1, or 2 hours, and hepatic MAPK levels were examined. We observed rapid induction of phospho-JNK1/2 10 minutes after TNF injection in both Mkp1+/− and Mkp1−/− mice (Figure 3A). This induction declined rapidly in Mkp1−/− mice, starting 0.5 hours after TNF injection, when phospho-JNK1/2 levels in liver clearly demonstrated that JNK2−/− mice were protected against TNF-induced inflammation, especially at the later 6-hour time point (Figure 4, C and D, and Supplemental Figure 5). Together, these data indicated that JNK2 has a proinflammatory role.

Because GRdim/dim mice were very sensitive to TNF and had weaker induction of MKP1, it is conceivable that these mice also display stronger activation of JNK1/2 upon TNF challenge. Therefore, we evaluated phospho-JNK1/2 levels 0.5 hours after TNF challenge in GRwt/wt and GRdim/dim mice. Interestingly, activation of JNK1/2 was stronger in GRdim/dim mice (Figure 3B), as was observed in Mkp1−/− mice. These observations indicated that activation of JNK1/2 is stronger in Mkp1−/− and GRdim/dim mice.
can be partly rescued by specifically inhibiting JNK2. We therefore conclude that JNK2 is one of the critical players responsible for the increased sensitivity of Mkp1–/– mice to TNF.

Furthermore, we assessed the response of GR dim/dim Jnk2–/– mice to shock. Control GR wt/wt Jnk2+/+, GR dim/dim, and GR dim/dim Jnk2–/– mice were injected i.p. with 20 μg TNF (which is lethal for GR dim/dim mice with a mixed C57BL/6-FvB background), and mortality and body temperature were monitored. The sensitivity of GR dim/dim Jnk2–/– mice was intermediate between those of GR wt/wt Jnk2+/+ and GR dim/dim mice (Figure 5, E and F). These results suggest that JNK2, at least in part, rescues the sensitivity of GR dim/dim mice to TNF, as it did in Mkp1–/–Jnk2–/– mice. Taken together, these data indicate that dimerized GR protects against TNF-induced shock by induction of MKP1 and subsequent inhibition of JNK2.

Discussion

TNF is a pleiotropic cytokine with strong proinflammatory capacity, and it is a well validated drug target in several inflammatory disorders, such as rheumatoid arthritis, IBD, and psoriasis (1, 2). However, the use of biological drugs inhibiting TNF is very expensive and can result in unwanted side effects, such as tuberculosis (3).

It has been known for a long time that GCs protect against TNF-induced lethal shock and that this protection is mediated by binding of GCs to GR, a very potent antiinflammatory protein (7). However, therapy with GCs can also induce side effects, such as diabetes. It is believed that the side effects are induced by TA, whereas the antiinflammatory effects are mainly attributable to the TR actions of GR. Most studies claim that interaction of monomeric GR with transcription factors forms the basis for its antiinflammatory activ-
The Journal of Clinical Investigation

Figure 6

JNK2 is an essential player in TNF lethality, leading to cell death, bowel damage, intestinal inflammation, systemic inflammation, and consequent death. In GR\textsuperscript{wt/wt} mice, TNF can induce Mkp1 via GR dimers. Mkp1 dephosphorylates JNK2 and hence inhibits its activity. In GR\textsuperscript{dim/dim} mice, TNF fails to induce Mkp1. These mice show prolonged and higher activation of JNK2, which results in a higher sensitivity to TNF- mediated shock. Dashed lines indicate the possible involvement of other MKP1 targets in the TNF pathway.

TNF-induced shock in Mkp1\textsuperscript{+/+} mice. A functional Mkp1 gene is probably required for maximal protective effects, as previously shown for DEX protection against endotoxic shock (40). In that study, DEX only partially protected Mkp1\textsuperscript{+/+} mice from endotoxic shock, whereas WT mice were completely protected from endotoxin-induced mortality. Our present results indicated that DEX pretreatment, in the absence of MKP1, did not fully protect Mkp1\textsuperscript{+/+} mice from the lethal effect of TNF. Thus, MKP1 was required for the protection provided by GCs against TNF. However, TNF-induced mortality was somehow delayed in DEX-versus solvent-treated Mkp1\textsuperscript{+/+} mice. Despite the essential role of MKP1 in the protective effects of GCs in several inflammatory models, including our TNF model, some GC effects occur independently of MKP1. GCs might have some direct antiinflammatory effects, or they might also act via the induction of other antiinflammatory proteins. This idea is supported by the prior finding that inhibition of protein synthesis in bone marrow–derived mast cells from Mkp1\textsuperscript{+/+} mice partly reverses the antiinflammatory effects of GCs (41).

In keeping with the well-known dephosphorylation actions of MKP1, we showed here that Mkp1\textsuperscript{+/+} mice exhibited prolonged or TNF, which suggests that Mkp1 might play a role in the protection against TNF. Several studies have shown that MKP1 protein levels parallel mRNA levels. Valledor et al. showed that synthesis of MKP1 protein is tightly correlated with the time course of Mkp1 mRNA expression in LPS-treated macrophages (33). MKP1 protein levels were also found to follow the same pattern of induction as Mkp1 mRNA in H\textsubscript{2}O\textsubscript{2}-treated HeLa cells (34). We also measured MKP1 protein levels in our model, and our data showed that MKP1 protein levels indeed followed Mkp1 mRNA expression levels. Thus, the induction of Mkp1 mRNA expression and MKP1 protein levels both relied on GR dimerization.

The regulation of MKP1 is of much interest, but remains controversial. Recently, ChIP sequencing studies revealed a GRE site in the promoter region of Mkp1 (35, 36). Furthermore, it was shown that DEX treatment leading to Mkp1 induction in liver and in various other cell types is dependent on GR dimerization (37). This was confirmed by our ChIP results, which indicated that binding of GR to Mkp1 and subsequent Mkp1 induction required GR dimerization. However, this was at odds with previous findings showing that in GR\textsuperscript{dim/dim} macrophages, Mkp1 can still be induced by DEX (17). Tissue specificity could be the reason for this discrepancy.

The prominent role of Mkp1 in the control of the excessive inflammation induced by TNF was confirmed in Mkp1\textsuperscript{+/+} mice, which were extremely sensitive to TNF compared with Mkp1\textsuperscript{+/+} mice. These findings correlated with the antiinflammatory effects of MKP1 described in other inflammation models (17–19).
JNK1/2 phosphorylation. Moreover, GRdim/dim mice had higher levels of phospho-JNK1/2. To determine whether increased JNK activation indeed forms the basis of the hypersensitivity of Mkp1–/– and GRdim/dim mice to TNF, we studied the effects of TNF in Jnk1–/– and Jnk2–/– mice. We found that Jnk2–/– mice, but not Jnk1–/– mice, were resistant to TNF. The absence of JNK2 led to resistance against very early TNF-induced gut epithelial cell death, intestinal inflammation, and intestinal permeability as well as against subsequent systemic inflammation. We previously demonstrated that bowel damage is a very fast and essential step in TNF-induced lethal inflammation (42, 43), and other groups have shown that TNF mediates gut epithelial cell death in genetic mouse models of IBD, such as in the IKKγ knockout mouse (44). JNK molecules have been shown to mediate the apoptosis activity of TNF (45, 46), and JNK2 has been shown to induce apoptosis by regulating both mitochondrial and lysosomal death pathways (24, 25). Furthermore, several studies have indicated a role for JNK in regulating apoptosis in IECs (47, 48). Additionally, Ray et al. showed increased levels of both phospho-JNK1 and phospho-JNK2 in IEC-6 cells upon TNF challenge when the cells were also treated with MKP1 siRNA (48). That JNK increase subsequently resulted in more apoptosis in these cells, mediated by activated caspase 9 and caspase 3. Phospho-JNK1/2 levels were induced in IEC-6 cells within 15 minutes of TNF treatment and declined after 1 hour. This is in accordance with our present results using liver samples.

Here, we provided evidence that JNK2, but not JNK1, was an essential player in mediating apoptosis of IECs in response to TNF. We therefore conclude that JNK2 is a critical mediator of TNF lethality. Our data support the idea that JNK2 plays an essential role in the in vivo effects of TNF by mediating early cell death of IECs, which leads to disruption of the intestinal barrier and consequent systemic inflammation.

Since Mkp1–/–Jnk2–/– and GRdim/dimJnk2–/– mice were significantly less sensitive to TNF than were Mkp1–/+ and GRdim/dim mice, respectively, the hypersensitivity of Mkp1–/– and GRdim/dim mice was largely due to JNK2. Since Mkp1–/–Jnk2–/– mice did show the same extent of intestinal damage as control mice, JNK2 seemed to be critical for induction of intestinal damage. This is confirmed by another study showing that JNK mediates apoptosis of IECs (48). Furthermore, the authors show that knockdown of MKP1 in IECs by using siRNA enhances the activity of JNK and increases subsequent apoptosis. However, in our study, the response to TNF in Mkp1–/–Jnk2–/– mice was intermediate between those of Mkp1–/–Jnk2–/– and Mkp1–/+ mice. Thus, it is likely that the TNF pathway involves other players contributing to its lethal effects, especially its inflammatory effects, such as MAPK p38 and ERK. p38 in particular seems to be an excellent candidate, because the substrate specificity of MKP1 is greater for p38 and JNK than for ERK (18, 19). Moreover, several studies have proven an important role for p38 in mediating acute inflammation, such as the successful use of a MAPK inhibitor in vivo by Bhattacharyya et al. (49). These authors showed that a specific p38 inhibitor protected macrophage-specific GR–/– mice from LPS lethality attributed to impaired induction of MKP1. Furthermore, a critical role for p38 and subsequent NF-κB activation was recently described in several reports. It was shown that curcumin, which has anti-inflammatory and antioxidant activities, protects the intestinal mucosal barrier by induction of MKP1 and subsequent inactivation of p38 and of NF-κB-mediated transcription (50). In addition, some reports suggest that ERK could be an important mediator of TNF-induced shock and provide a link with MKP1 (51, 52). However, because we observed identical phospho-p38 and phospho-ERK levels in Mkp1–/+ and Mkp1–/– mice, the contribution of these MAPKs in the context of the present study can be ruled out. It is probable that other yet-unidentified targets of MKP1 could account for the TNF-induced toxic effects. Taken together, our results suggest that by limiting the duration of JNK2 activity, MKP1 provides a crucial negative feedback mechanism of GCs during inflammatory responses.

In summary, we showed that dimerization of GR was essential for protection against acute TNF-mediated inflammation and critical for Mkp1 induction, and hence controlled activation of the proapoptotic JNK2. Our observations suggest that JNK2 mediates TNF lethality by inducing intestinal apoptosis and inflammation as well as subsequent systemic inflammation (Figure 6). To our knowledge, this is the first in vivo evidence that GR dimerization is important in the regulation of TNF-induced apoptosis.

Another important conclusion of our work is that both anti-apoptotic and anti-inflammatory effects of GCs necessitate the induction of gene expression and require GR dimerization. Thus, the dissociation model calling for prevention of GR-induced gene induction and stimulation of GR transrepression by synthetic GR ligands (i.e., selective GR agonists [SEGRAs]), although attractive, has some limitations, at least in this type of acute inflammation. Especially in acute inflammatory settings, GR-induced gene expression makes a major contribution to protection, and hence inhibition of GR dimerization might be dangerous.

Methods

Mice. C57BL6/J mice were purchased from Janvier. GRdim mice were generated by Reichardt et al. (21) and backcrossed on a FVB/N background. Heterozygous GRdim mice were intercrossed to generate GRdim/dim homozygous mutant mice and GRwt/wt homozygous WT mice. Mkp1–/– mice had a C57BL6/J background and were generated by Bravo et al. (53) and provided by Y. Liu (Ohio State University, Columbus, Ohio, USA). Jnk1–/– and Jnk2–/– mice had a C57BL6/J background and were purchased from the Jackson Laboratories. Pure C57BL6/J mice were used as controls for Mkp1–/–Jnk1–/– and Jnk2–/– mice. In order to generate Mkp1–/–Jnk2–/– and GRdim/dimJnk2–/– mice, single knockouts were crossed, and F1 hybrids were intercrossed to yield double-WT Mkp1–/–Jnk2–/– and GRwt/wtJnk2–/– mice and double-deficient Mkp1–/–Jnk2–/– and GRdim/dimJnk2–/– mice. All offspring were genotyped by PCR on genomic DNA isolated from tail biopsies. The mice were kept in individually ventilated cages under a 12-hour dark/12-hour light cycle in a conventional animal house and received food and water ad libitum. All mice were used at 8–12 weeks of age.

Reagents. Recombinant mouse TNF was produced in E. coli and purified to homogeneity in our laboratories. TNF had a specific activity of 1.2 × 10⁸ IU/mg and no detectable endotoxin contamination.

Injections and sampling. TNF was diluted in pyrogen-free PBS. All injections were given i.p. Blood was withdrawn with a glass capillary from the retroorbital plexus and allowed to clot overnight at 4°C. The clot was then removed, and serum was collected after centrifugation at 20,000 g for 10 minutes and stored at –20°C. For sampling liver tissue, mice were killed by cervical dislocation, and tissue samples were fixed briefly in 4% paraformaldehyde and embedded in paraffin by a standard protocol (Tissue-Tek VIP, Sakura). A piece of liver was stored in RNA later (Qiagen) for RNA preparation, and the rest was snap-frozen in liquid nitrogen and stored at –80°C.

Measurements of cytokines in circulation. Serum IL-6 was determined with a 7TD1 bioassay (54). Serum samples were also assayed for several cytokines using Luminox technology (Bio-Rad, Nazareth-Eke) following the manufacturer’s protocol.
RNA was isolated with the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was measured with Nanodrop 1000 (Thermo Scientific), and 1 μg RNA was used to prepare cDNA with the PCR purification kit (Qiagen). qPCR was performed using the Roche LightCycler 480 system (Applied Biosystems). MKP1 primer pairs were as follows (based on ref. 56): MKP1-1 (1.3), 5′-CAAGAGCTGAAGGAGCCCA-3′ and 5′-CAAGAGCTGAAGGAGCCCA-3′; MKP1-2 (2.4), 5′-GTGCTGGTGCTGGCTCATCGAAT-3′ and 5′-GGGCTCACAGCCTGGTCCCTC-3′; MKP1-3 (2.8), 5′-GCTTCCAGAGGTGCGCAAG-3′ and 5′-CAGGAGCTTGGTTGCTTGTG-3′.

Intestinal permeability. Animals underwent an in vivo intestinal permeability assay at 8 hours after TNF treatment using methods described previously (57). At 3 hours after TNF, 200 μl FITC-dextran (25 mg 4.4-kDa FITC-dextran in 1 ml PBS) was administered orally. At 8 hours after TNF, blood was collected into heparinized Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes. Plasma was collected and subsequently assayed for concentration of FITC-dextran.

Statistics. Survival curves (Kaplan-Meier plots) were compared by log-rank test, and final outcomes by χ2 test. Statistical significance of differences between groups was evaluated by 2-tailed Student’s t tests with 95% confidence intervals and by 2-way ANOVA. All data are expressed as mean ± SEM. P values less than 0.05 were considered significant.

Study approval. All animal experiments were approved by the institutional ethics committee for animal welfare of the Faculty of Sciences, Ghent University.